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C1q, the recognition subcomponent of the classical pathway of complement, drives microglial activation

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Abstract

Microglia, central nervous system (CNS) resident phagocytic cells, persistently police the integrity of CNS tissue and respond to any kind of damage and pathophysiological changes. These cells sense and rapidly respond to danger and inflammatory signals by changing their cell morphology, by release of cytokines, chemokines or nitric oxide, and by changing their MHC expression profile. We have shown previously, that microglial biosynthesis of the complement subcomponent C1q may serve as a reliable marker of microglial activation ranging from undetectable levels of C1q biosynthesis in resting microglia to abundant C1q expression in activated, non-ramified microglia. In this study, we demonstrate that cultured microglial cells respond to extrinsic C1q with a marked intracellular Ca²⁺ increase. A shift towards proinflammatory microglial activation is indicated by the release of IL6, TNF- α , and nitric oxide and the oxidative burst in rat primary microglial cells, an activation and differentiation process similar to pro-inflammatory response of microglia to exposure to LPS. Our findings indicate (i) that extrinsic plasma C1q is involved in the initiation of microglial activation in the course of CNS diseases with blood brain barrier impairment and (ii) C1q synthesized and released by activated microglia is likely to contribute in an autocrine/paracrine way to maintain and balance microglial activation in the diseased CNS tissue.

Keywords

microglia; MBL; C1q; Calcium increase; TNF-alpha; IL6; proliferation

INTRODUCTION

Microglial cells are intrinsic components of the central nervous system (CNS) and act as sensors of pathological changes in CNS tissue. In the normal, healthy brain resting microglial cells show a ramified morphology, but during any kind of disease or pathological event such as after trauma, stroke or in multiple sclerosis, they transform into an activated,

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amoeboid form. They can proliferate, present antigens, release a broad range of substances such as cytokines, chemokines and nitric oxide (NO) and migrate to the site of injury or damage. Microglial activation is triggered by 'on' or 'off' signals, i.e. factors which newly appear or strongly increase during the pathologic event, or by factors which are constitutively present and decrease in response to pathology (Hanisch and Kettenmann, 2007). Components of the complement system are candidates to mediate such 'on' signals for microglia, since blood constituents can enter the brain after damage.

The complement system consisting of more than 30 plasma and associated cell membrane proteins serves as a component of both innate and adaptive immunity. Activation of the complement system results in a broad change of cellular responses of diverse cell types, such as B and T cell differentiation (Reid *et al.*, 1995), induction of neuronal cell death (Shen *et al.*, 1997, Morgan *et al.*, 1996) and the neutrophil chemotaxis and the induction of oxidative burst of neutrophils (Leigh *et al.*, 1998). Complement activation can be triggered via three different pathways, the classical pathway (CP), the alternative pathway (AP) and the recently discovered lectin pathway (LP) (Schwaebble *et al.* 2002). The three pathways converge by generation of a C3 convertase, which mediates cleavage of C3, followed by activation of the terminal complement pathway and the formation of the membrane attack complex (MAC) (Roos *et al.*, 2004).

The classical activation pathway of complement is initiated by binding of the classical pathway recognition molecule C1q to targets such as immune complexes which in turn leads to the activation of the C1q associated serine protease C1r which cleaves and activates its substrate C1s. Activated C1s that initiates activation of the downstream complement cascade by cleavage of C4 and C2 to form the C3 convertase complex C4b2a. C1q is a macromolecule of approximately 460 kDa, composed of six hetero-timers formed of the C1q A-chain, B-chain and C-chain (encoded by 3 clustered homologous genes) forming a characteristic "bunch of tulips" structure (Schwaebble *et al.*, 1995). Each chain is composed of a C-terminal globular domain (forming the "tulip head") followed by an extended collagen-like sequence (forming the "tulip stalk"). We have previously shown that C1q is exclusively synthesised by myeloid cells and cells of the monocyte macrophage lineage, including peripheral blood monocytes, tissue resident macrophages, follicular dendritic cells and by CNS resident and activated microglial cells (Müller *et al.*, 1978, Dietzschold *et al.* 1995; Schwaebble *et al.* 1995; 2005; Schäfer *et al.* 2000; Lynch *et al.* 2004).

MBL is one of several recognition subcomponents of the lectin activation pathway of complement. MBL shows a very high degree of structural similarity to C1q and forms a macromolecule of a very similar shape to that of C1q. The subunits, however, are homotrimers composed of a C-terminal C-type lectin domain preceded by an extended collagen-like sequence. The collagen-like domains of both MBL and C1q are thought to bind to identical cellular receptors for both C1q and MBL, and although several candidate receptors for C1q and MBL have recently been proposed, only two of these candidate receptors were shown to exhibit binding for both under physiological conditions (Ogden *et al.* 2001, Zutter and Edelson 2007).

While C1q is predominantly synthesized by peripheral tissue macrophages and dendritic cells, MBL biosynthesis mainly, but not exclusively, occurs in the liver (Wagner *et al.* 2003, Lu *et al.*, 2007). C1q and MBL can reach levels of more than 35 µg/ml at damaged sites (Lindgren *et al.*, 1984) and may act as chemoattractants for neutrophils, eosinophils and mast cells (Vegh *et al.*, 2006).

In the present study, we explored whether extrinsic C1q modulates the activation of microglia and increases the release of proinflammatory cytokines IL6 and TNF- α by using an established cell culture model of microglial activation.

MATERIAL AND METHODS

Isolation and purification of rat C1q/MBL

Rat C1q was purified from whole rat serum using a protocol adapted from that described by Yonemasu and Sasaki 1981. Briefly, 100 ml serum was dialyzed against 3×2 L of 10 mM EGTA over 48 hours. The resulting precipitate was recovered by centrifugation at $20000 \times g$, washed in 10 mM EGTA followed by repeated centrifugation. The washed pellet was redissolved in 5 ml of 25 mM HEPES pH 8.0, 500 mM NaCl, 10 mM EDTA and applied in batches of 1 ml onto a Superose 6 size-exclusion column. Fractions eluted from the column were visualized by SDS-PAGE and also by Western Blotting using rabbit anti-rat C1q polyclonal antiserum. C1q-containing fractions were pooled and dialyzed overnight against 25 mM HEPES pH 7.4, 100 mM NaCl, 10 mM EDTA and subsequently incubated with 5 ml human non-immune IgG-Sepharose for 45 minutes with stirring. The affinity Sepharose was then loaded into a column case, washed with 50 ml of 25 mM HEPES pH 7.4, 100 mM NaCl, 10 mM EDTA before eluting with 20 ml of 100 mM CAPS pH 11.2, 1 M NaCl, 10 mM EDTA. The eluant was quickly dialyzed against 20 mM Na-Acetate pH 5.4, 50 mM NaCl, 10 mM EDTA and loaded onto a Mono-S cation exchange column that was eluted using a 50-500 mM gradient of NaCl. C1q characteristically emerges late in the elution profile as a single peak. Integrity and purity of the C1q - assessed as 95% - was confirmed by Coomassie-staining of SDS-PAGE, and also Western blotting. Recombinant rat MBL was produced from CHO cells and purified on mannose-Sepharose affinity columns as described in Wallis and Drickamer 1999.

Microglial cell culture

Microglial cultures were prepared from brains of newborn Wistar rats as described previously (Prinz *et al.*, 1999). In brief, the forebrain was carefully freed of larger, superficial blood vessels and meninges. Cortical tissue was trypsinized for 2 min, dissociated with a fire-polished pipette and washed twice. Mixed glial cells were cultured for 9 to 12 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and antibiotics, with medium changes every third day. Microglial cells were then separated from the underlying astrocytic monolayer by gentle shaking of the bottles for one hour at 37°C in a shaker-incubator (100 rpm). The cells were seeded on glass coverslips or 96-well plates at a density of 5×10^4 cells/well. Cultures usually contained > 95 % microglial cells which can be stained with Griffonia simplicifolia isolectin B4 (Sigma, Deisenhofen, Germany), a marker for microglia. Cultures were used for experiments 1- days after plating. Cell media and supplements were purchased from Seromed/Biochrom (Berlin, Germany).

Calcium imaging of cultured microglia

Loading of microglial cells on glass coverslips with the calcium indicator Fura-2 AM (5 μM) was achieved by incubation for 45 to 60 min at 37°C in the presence of the detergent 0.02 % Pluronic F-127. Equilibration was made possible by placement of coverslips in a perfusion chamber on the stage of an inverted microscope (Axiovert FS, Zeiss, Oberkochen, Germany) for 10 min at room temperature. C1q (50 $\mu\text{g/ml}$) and MBL (50 $\mu\text{g/ml}$) were applied by local application pipette and for measurement of intracellular calcium changes. The Ca^{2+} -sensitive fluorescent dye Fura-2 AM was excited at two alternating wavelengths of 340 ± 5 nm and 380 ± 5 nm. Excitation was carried out by employing a monochromator (TILL Photonics, München, Germany). The emitted light was collected at 530 ± 10 nm by a

cooled CCD camera. The monochromator and CCD camera were controlled by ICE (HEKA electronics, Lambrecht/Pfalz, Germany). Ratios were collected at 1 s time intervals. The change of intracellular calcium was calculated using the method established previously (Grynkiewicz *et al.*, 1985). At the end of an experiment, the application of ATP served as a control for the viability and responsiveness of the cells.

Chronic stimulation and NO, IL-6 and TNF-alpha quantification

Microglia (plated in 96 well plates, 10^6 cells per well) were stimulated for 24 h with C1q and MBL (1 to 50 $\mu\text{g/ml}$) alone and in combination with LPS (100 ng/ml; Sigma). Analysis of NO in the supernatant was performed by using Griess reagent as described in Färber *et al.*, 2005. Analysis of IL-6 and TNF-alpha contents in the supernatants was carried out in accordance with the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). The colorimetric reaction was analyzed in a microplate reader (SLT, Spectra Lab Instruments Deutschland GmbH, Crailsheim, Germany) as absorbance at 450 nm with 540 nm as reference.

Determination of microglial cell proliferation by using Cresyl Violet assay

Microglia cells (plated in 96 well plates, 10^6 cells per well) were treated for 24 h with C1q or MBL (1 to 50 $\mu\text{g/ml}$) alone or in combination with LPS (100 ng/ml; Sigma) at 37 °C under 5 % CO_2 . Microglial proliferation was determined by the crystal violet assay as described in Wosikowski *et al.*, 1993.

LHD assay for analysing microglial cell death

Microglial cells (plated in 96 well plates, 10^6 cells per well) were treated for 24 h with C1q or MBL (1 to 50 $\mu\text{g/ml}$) at 37 °C under 5 % CO_2 . Cell death of microglial was measured by using LDH assay (Roche) by following the manufacturer protocol.

Lactate dehydrogenase - Cell viability test

Lactate dehydrogenase (LDH) - Cytotoxicity Detection Kit (Roche Molecular Biochemicals) has been used for quantification of cell death and cell lysis, according to the manufacturer's instructions. LDH test is a colorimetric assay based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. In our experiments, we analysed the LDH (lactate dehydrogenase) activity after stimulation of microglial cells with C1q (1 to 50 $\mu\text{g/ml}$) and MBL (1 to 50 $\mu\text{g/ml}$) for 24 h in cell supernatants.

Oxidative burst assay

The microglia cells plated in 96 well plates were treated for 24 h with either C1q or MBL (1 to 50 $\mu\text{g/ml}$) and C1q in combination with LPS (100 ng/ml; Sigma) in 37 °C under 5 % CO_2 . Subsequently, Dihydrofluorescein (to a final concentration of 20 μM) was added and incubated for further 3 h in 37 °C under 5 % CO_2 . The colorimetric reaction was quantified in a microplate reader (SLT, Spectra Lab Instruments Deutschland GmbH, Crailsheim, Germany) reading the absorbance at 405 nm with 490 nm as reference.

Phagocytosis assay

Microglial cells were plated on coverslips at a cell density of 10^5 cells/coverslip and were stimulated with MBL (50 $\mu\text{g/ml}$) or DMEM/10 % FCS (as a negative control) for 30 min at 37°C. Opsonized microparticles for phagocytosis were applied at concentration of 10^8 particles/ml. Equivalent sample were incubated at 4°C. After 30 min, the reaction was stopped by washing the cells twice with ice cold PBS. The cells were washed twice to remove the non phagocytosed particles. The cells were fixed for 30 min using 4 % paraformaldehyde and were analysed by counting the phagocytosed particles to determine

the phagocytosis index, expressing phagocytic activity as percentage of phagocytes with particle inclusions multiplied by the number of particles contained. Based on 100 microglial cells per treatment, number of phagocytosing cells were analysed for number of included particles and classified on different phagocytose grades: grade 1 (1-4 particles), 2 (5-7 particles), 3 (8-10 particles), 4 (> 10 particles). The overall phagocytic activity for each assay is expressed using the phagocytosis index.

RESULTS

C1q and MBL differentially *trigger* TNF- α and IL6 release in microglia cells

Microglial activation by inflammatory stimuli leads to release of IL6 and TNF- α . To test whether C1q or MBL would serve as a trigger for such microglial activation, we stimulated microglia cells over 24 h with rat C1q (1 to 50 μ g/ml) or rat MBL (1 to 50 μ g/ml), measured the release of IL6 and TNF- α in the supernatant and compared it to stimulation with LPS (100 ng/ml). Defining the LPS stimulated IL6 and TNF- α release at 100 %, C1q (1 to 50 μ g/ml) triggered even a stronger release of IL6, namely up to 141 % (at 50 μ g/ml, n = 3) and a similar release of TNF- α , namely 94 % (at 50 μ g/ml, n = 6; Figure 1 A-C). By contrast, MBL (1 to 50 μ g/ml) triggered a lower release of IL6, namely 37 % (at 25 μ g/ml, n = 3) and by 25 % (at 50 μ g/ml, n = 3) while lower concentrations failed to induce a significant release (Figure 1 F). In addition, MBL (1 to 50 μ g/ml) did not significantly increase TNF- α release at any of the concentrations tested (Figure 1 E). We also tested whether C1q would affect the LPS stimulated TNF- α and IL6 release. Co-stimulation of C1q (1 to 50 μ g/ml) with LPS over 24 h did not increase LPS-induced IL6 release (Figure 1 D), but decreased the release of TNF- α at concentrations of 25 μ g/ml (to 78 %, n = 3) and 50 μ g/ml (to 72 %, n = 3) (Figure 1 B).

C1q attenuated, while MBL increased microglial proliferation

To test the effect of rat C1q and rat MBL on microglial proliferation we used a Cresyl violet staining assay (18). We incubated microglial cells in 96 well plates with C1q (1 to 50 μ g/ml) and MBL (1 to 50 μ g/ml) alone and C1q in combination with LPS (100 ng/ml) over 24 h and analysed the proliferation rate. Incubation with C1q alone significantly attenuated microglial proliferation in a dose dependent manner (at 1 μ g/ml to 73 % and at 50 μ g/ml to 38 %, n = 3; Figure 2 A) as compared to the control (i.e. medium only set at 100 %). MBL had no effect at low concentrations, but increased microglial proliferation at higher concentrations (25 μ g/ml to 141 % and at 50 μ g/ml to 153 %, n = 3; Figure 2 B).

LPS leads to a decrease in microglial proliferation as previously described (Ganter *et al.* 1992) (Figure 2 A). Upon co-stimulation with LPS (100 ng/ml), C1q further attenuated microglial proliferation except at high concentration of 50 μ g/ml to 53 %, n = 6, comparing to LPS (72 %, n = 3). Lower C1q concentrations (< 50 μ g/ml did not have a significant effect (data not shown).

C1q increase nitric oxide release and oxidative burst

The release of nitric oxide (NO) and the production of reactive oxygen species is another microglial activation parameter triggered by LPS (Zielasek *et al.*, 1992). We therefore analysed the effect of rat C1q and rat MBL on the release of NO using the Griess reagent and tested the production of reactive oxygen species using an oxidative burst assay. Upon stimulating microglial cells with C1q and MBL and measuring the release of NO in the supernatant, we found, that C1q (1 to 50 μ g/ml) increased the release of NO at 5 μ g/ml (to 98 %) and higher concentrations (50 μ g/ml to 148 %) as compared to LPS alone (100 ng/ml, 100 %) (Figure 3 A, n = 3). C1q alone increased the production of reactive oxygen species (at 1 μ g/ml to 137 % and at 50 μ g/ml to 155 %) to a similar extent as LPS (100 ng/ml to 126

%) (Figure 4 A, n = 3). In contrast MBL (1 to 50 µg/ml) was ineffective at inducing either oxidative burst (Figure 4 C) or NO release (Figure 3 C). When we analysed LDH activity in the supernatant for quantification of cell death induced by MBL (1 to 50 µg/ml) and C1q (1 to 50 µg/ml) we found, that MBL increased LDH activity (at 1 µg/ml to 132 % up to 50 µg/ml to 140 %, n = 3), while C1q did not.

Co-stimulation of C1q with LPS only slightly, but significantly increased NO release at a concentration of 25 µg/ml (to 116 %) as compared to LPS alone (100 %) (n = 3, Figure 3 B). The oxidative burst, in contrast, is effectively increased by C1q in concert with LPS (at 1 µg/ml to 155 % and at 50 µg/ml to 174 %) compared to LPS alone (126 %, n = 3, Figure 4 B).

MBL induces microglial phagocytosis

C1q has been shown to increase phagocytic capacity of rat microglial cells (Webster *et al.*, 2000), a process involving CD93 (formerly known as C1qRp) which by itself appears not to bind to C1q directly (McGreal *et al.* 2002). In order to assess a possible effect of MBL on the phagocytic activity of stimulated microglial cells, we incubated pure recombinant rat MBL (50 µg/ml) over a time period of 30 min at 37°C with cultured microglial cells and measured their phagocytotic activity. We observed that MBL significantly increased microglial phagocytosis (to 152 %, as compared to the untreated control, n = 700, see Figure 5).

C1q but not MBL induced a transient calcium increase in microglial cells

To test, whether all microglial cells in a given culture are responsive to C1q or MBL we studied the Ca²⁺ responses of individual microglial cells after application of C1q or MBL. After loading of cultured rat microglia with the Ca²⁺-sensor fura-2, rat C1q (50 µg/ml) and rat MBL (50 µg/ml) were applied for 30 sec ejected from a glass pipette.

C1q triggered a transient increase in the fluorescence ratio (340/380 nm) indicating an increase of intracellular [Ca²⁺] in more than half of the cells tested (55.9 %; n=179 (Figure 6). The induced fluorescence ratio increase ranged from 0.055 to 0.675 (mean 0.179 +/- 0.010). As a control, we applied ATP (which is known to trigger large transient increases in intracellular Ca²⁺).

To compare the calcium responses, induced by C1q in activated microglia, we stimulated microglial cells over 24 h with LPS (100 ng/ml), stimulated the cells with C1q (50 µg/ml) and measured the fluorescence ratio in response to C1q (50 µg/ml). Only 33.9 % (n = 180 cells) responded to C1q stimulation and the increase in the fluorescence ratio was considerably smaller (mean 0.167 +/- 0.009; data not shown). In separate experiments, MBL (50 µg/ml) was applied via local application for 30 sec. However, no change in intracellular [Ca²⁺] was observed in non-activated and LPS-activated cells (n = 293 and 109, respectively; data not shown). All cells tested responded to ATP.

DISCUSSION

Complement factors and their receptors not only play a role in the initiation and progression of complement-mediated host defense against microbial infection in the periphery, but may also contribute to the pathology of degenerative and inflammatory CNS diseases (Gasque *et al.*, 2000; Webster *et al.*, 2000, Dietzschold *et al.* 1995, Morgan *et al.*, 1996, Schäfer *et al.* 2000, Depboylu *et al.* 2005). Complement proteins are often associated with Alzheimer's pathology. For example C1q is found in senile plaques (Eikelenboom *et al.*, 1989, Ishii and Hage *et al.*, 1984, McGeer *et al.*, 1989, Rogers *et al.*, 1992, Eikelenboom *et al.*, 1982, McGeer *et al.*, 1992, Yao *et al.*, 1992). Recent work on C1q-deficient animals implied that

C1q may have a detrimental effect on the progression of CNS diseases, as for example, C1q deficiency in animal models of Alzheimer's disease results in a slower loss of neuronal markers indicating a better survival of neurons in absence of C1q (Fonseca *et al.*, 2004). Likewise, C1q appears to facilitate the progression of prion disease with a significantly delayed onset of prion disease in C1q deficient mice (Klein *et al.* 2001). In contrast, larger infarct volumes after ischemia have been reported in C1q-deficient animals (Ten *et al.*, 2005) indicating that C1q can also act as a protective factor. In this context, it is important to note that microglial activation can be quite distinct with respect to the pathologic event and its course as recently reviewed (Hanisch and Kettenmann, 2007).

Complement factors may leak into the CNS through a damaged brain barrier, but some complement components can also be synthesised by CNS resident cells. Biosynthesis of complement factors has been described in astrocytes (Levi-Strauss *et al.*, 1987, Gordon *et al.*, 1992, Rus *et al.*, 1992, Gasque *et al.*, 1992, Gasque *et al.*, 1993, Gasque *et al.*, 1995), oligodendrocytes (Gasque *et al.*, 1996) and neurons (Thomas *et al.*, 2000, Johnson *et al.*, 1992, Pasinetti *et al.*, 1992). However, the overwhelming evidence in both, rodents and primates, identifies microglial cells and monocyte/macrophage cells entering the CNS as sites of C1q synthesis in the CNS (Dietzschold *et al.* 1995, Schäfer *et al.* 2000, Lynch *et al.* 2004, Depboylu *et al.* 2005, Johnson *et al.*, 1992, Pasinetti *et al.*, 1992, Haga *et al.*, 1983). C1q is present at low levels in the normal brain but is dramatically upregulated after injury (Lynch *et al.*, 2004), ischemia (Schäfer *et al.* 2000) and virus infections (Dietzschold *et al.* 1995; Depboylu *et al.* 2005). MBL was detected in association with blood vessels in the brain tissue of both AD patients and control subjects while the levels were lower in the Alzheimer patients (Lanzrein *et al.*, 1998).

The biosynthesis of complement factors in brain tissue implies relevant physiological roles. C5a, for instance, controls several functional parameters of microglia such as their chemotaxis and migration (Nolte *et al.*, 1996), activity of outward potassium currents (Iltschner *et al.*, 1996) or intracellular calcium signalling (Möller *et al.*, 1997).

In the present study, we have compared the effects of two distinct initiators of the complement system, C1q and MBL on functional parameters of microglial cells. Our Ca^{2+} imaging data indicate that C1q activates receptor(s) linked to Ca^{2+} signalling, while MBL has no effect. We therefore propose that C1q and MBL utilise different receptors on microglial cells and that the receptor complex inducing calcium signalling in response to C1q is not recognising or signalling in response to extrinsic MBL.

As reviewed recently, microglial activation is not an all-or-none process. We have used LPS-induced pro-inflammatory activation of microglia as a positive control and measured the induced release of pro-inflammatory cytokines and NO as well as a reduction in microglial proliferation. Our results demonstrate that C1q mimics the action of LPS and can thus be viewed as pro-inflammatory trigger for microglia. Similar proinflammatory activities of extrinsic C1q on endothelial cells has most recently been reported (Oroszlán *et al.* 2007)

MBL on the other hand may have more subtle actions on the activation state of microglia. MBL may trigger some functions of microglial cells in association with pathological events as our data show an increase in the phagocytic activity and proliferation rate of microglia in response to MBL. Such an increase in microglial proliferation was observed in the facial nucleus after facial nerve axotomy (Angelov *et al.*, 1995) and phagocytosis by microglia was described in response to Alzheimer's disease (Webster *et al.*, 2000).

We have used LPS as a stimulus to induce proinflammatory microglial activation in absence and presence of C1q and observed that in presence of both, LPS + C1q, C1q may synergistically enhance oxidative burst (see Figure 5B). The finding that addition of

increasing concentrations of extrinsic C1q may augment the effects of LPS on microglial cells was somewhat surprising as in a previous study using human peripheral blood mononuclear cells C1q and MBL were shown to limit LPS-induced production of proinflammatory cytokines, including interleukin (IL)1 α and IL1 β , and to increase the secretion of cytokines IL10, IL1 receptor antagonist, monocyte chemoattractant protein-1 (MCP1), and IL6 (Fraser *et al.*, 2006). We did not include the results obtained by adding LPS and increasing concentrations of our pure recombinant MBL, as it is well known that MBL binds and neutralises a broad spectrum of LPS preparations and a suppressive effect that MBL may have on LPS-triggered signalling and cytokine release may simply be caused by MBL competing for LPS-binding (Devyatyarova-Johnson *et al.* 2000).

Although many candidate receptors for either or both, C1q and MBL have been reported, the nature and structure of these cellular receptors still remains to be elucidated. At present, the best studied candidate receptor for both is a macromolecular complex formed of cell surface calreticulin and CD91 (Ogden *et al.* 2001). Likewise, a novel $\alpha 2\beta 1$ integrin was shown to physiologically bind to both C1q and MBL and elicit signaling in mast cells (Edelson *et al.* 2006) while a previous candidate receptor triggering a phagocytic response to C1q on cells of the monocyte-macrophage lineage, a candidate receptor called C1qRp, was shown to fail to bind to C1q (McGreal *et al.* 2002).

In conclusion, our study has clearly shown that exogenous C1q evokes calcium signalling in microglial cells and triggers the release of the pro-inflammatory cytokines IL6 and TNF- α suggesting that i.) extrinsic plasma C1q is involved in the initiation of microglial activation in the course of CNS diseases with blood brain barrier impairment and (ii) C1q synthesized and released by activated microglia is likely to contribute in an autocrine/paracrine way to maintain and balance microglial activation in the diseased CNS tissue.

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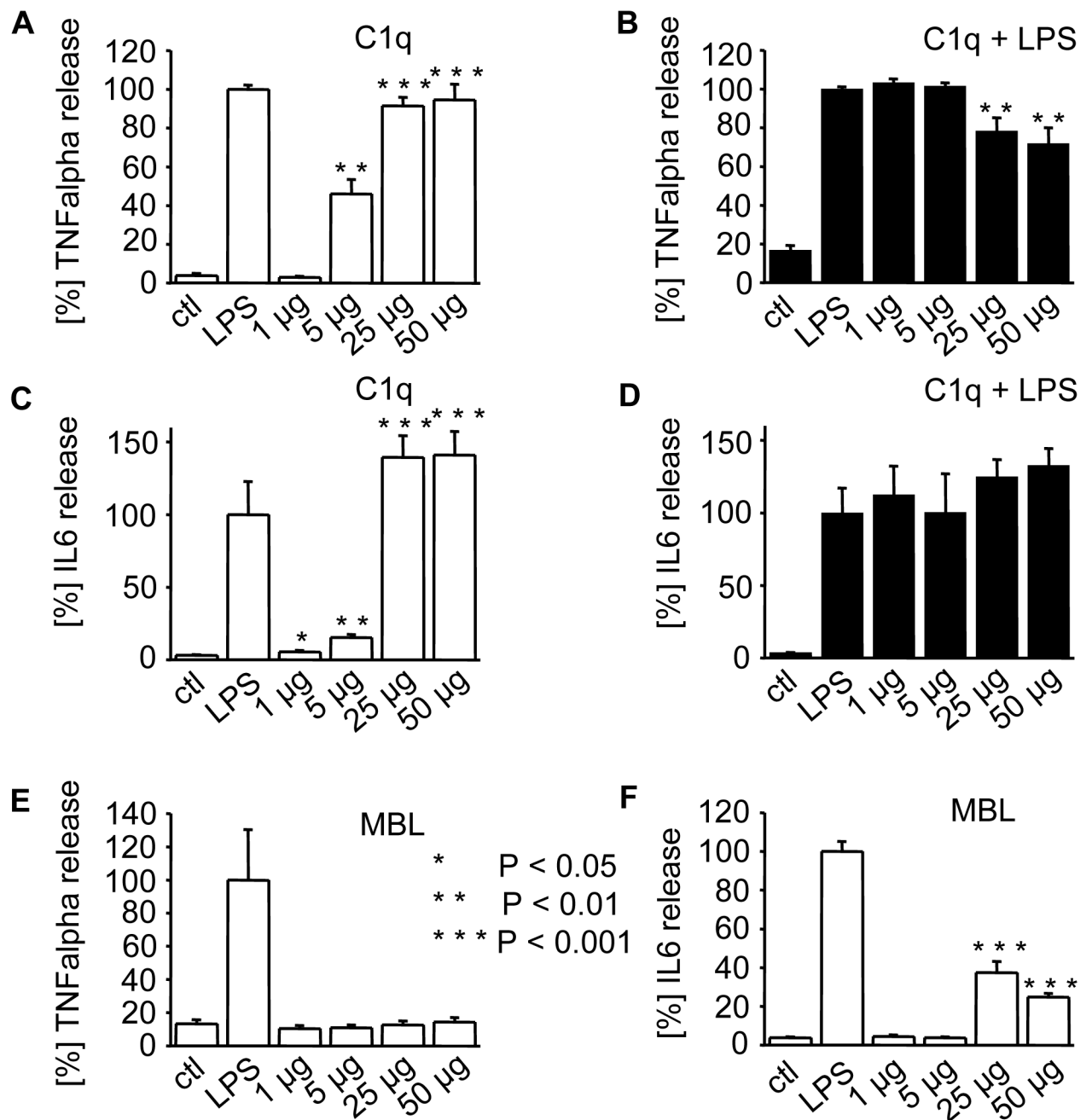
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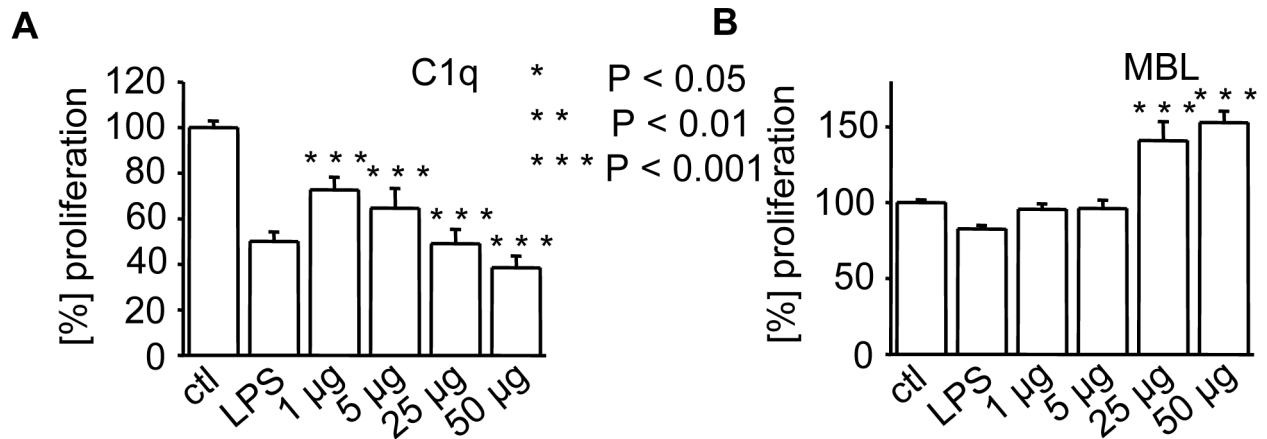
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**Figure 1.**

C1q and MBL regulate IL6 and TNF-alpha release in microglial cells in a defined pattern. Microglial cells were stimulated with C1q (A-D, 1 to 50 µg/ml) and MBL (E-F, 1 to 50 µg/ml) over 24 h alone and C1q in combination with LPS. The release of TNF-alpha and IL6 was measured in the supernatant and normalized to the response in the presence of LPS (100 %). "ctl" represents the release in the absence of LPS. Data are mean ± SEM summarized from three independent experiments.

**Figure 2.**

Proliferation of microglia cells is attenuated by C1q, but not by MBL.

Microglia cells were exposed to C1q (A, 1 to 50 µg/ml) and MBL (B 1 to 50 µg/ml). Proliferation was determined by using Cresyl Violet assay. The proliferation rate was standardized on DMEM/10 % FCS treated cells (ctl; 100 %). Data are mean ± SEM summarized from three independent experiments.

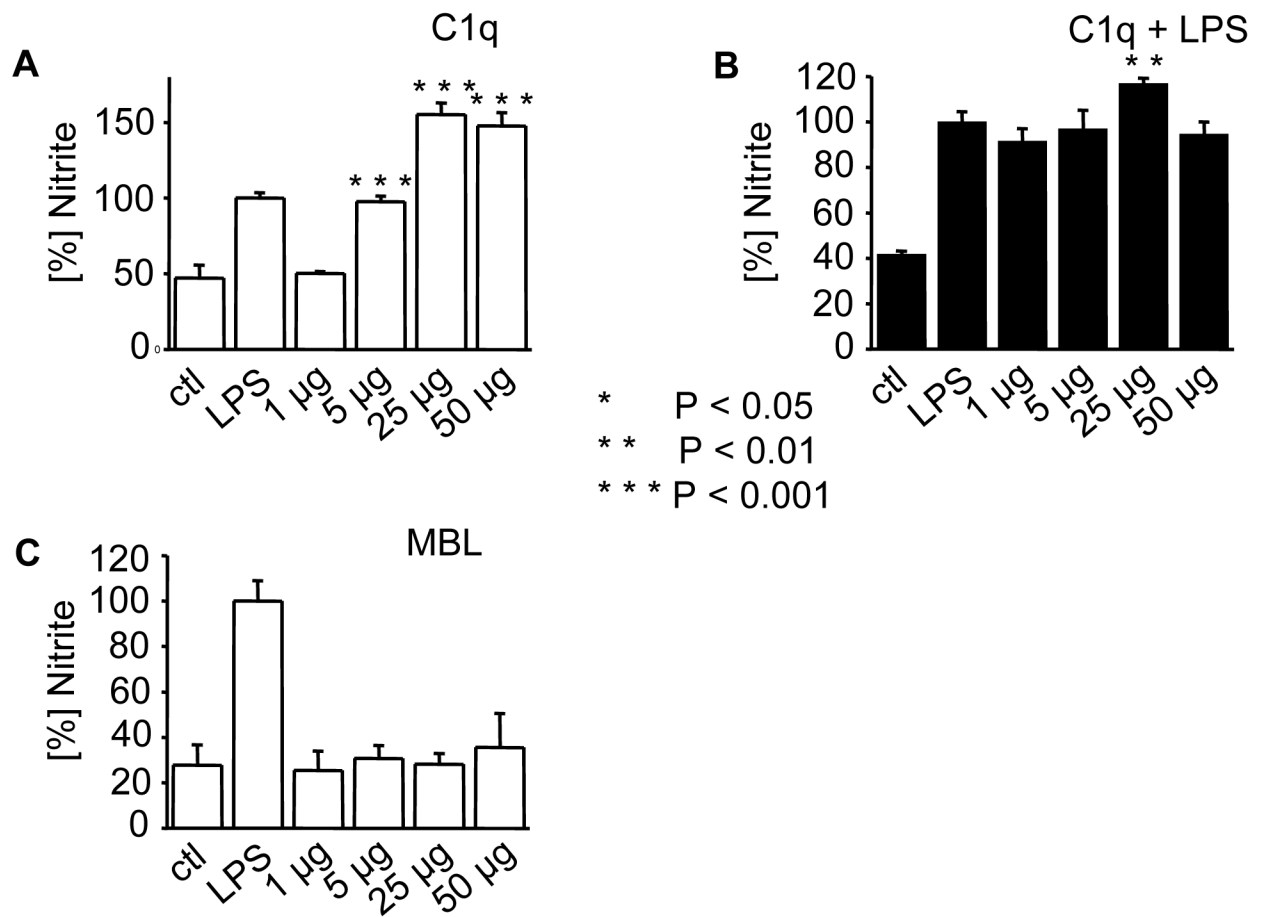


Figure 3.

LPS induced nitric oxide release is not attenuated by C1q

Microglia cells were stimulated with C1q (A, B, 1 to 50 μ g/ml) and MBL (C, 1 to 50 μ g/ml) alone and C1q in combination with LPS (100 ng/ml) over 24 h. The amount of nitrite in the supernatant was determined after 24 h using the Griess reagent. Data are mean \pm SEM summarized from three independent experiments.

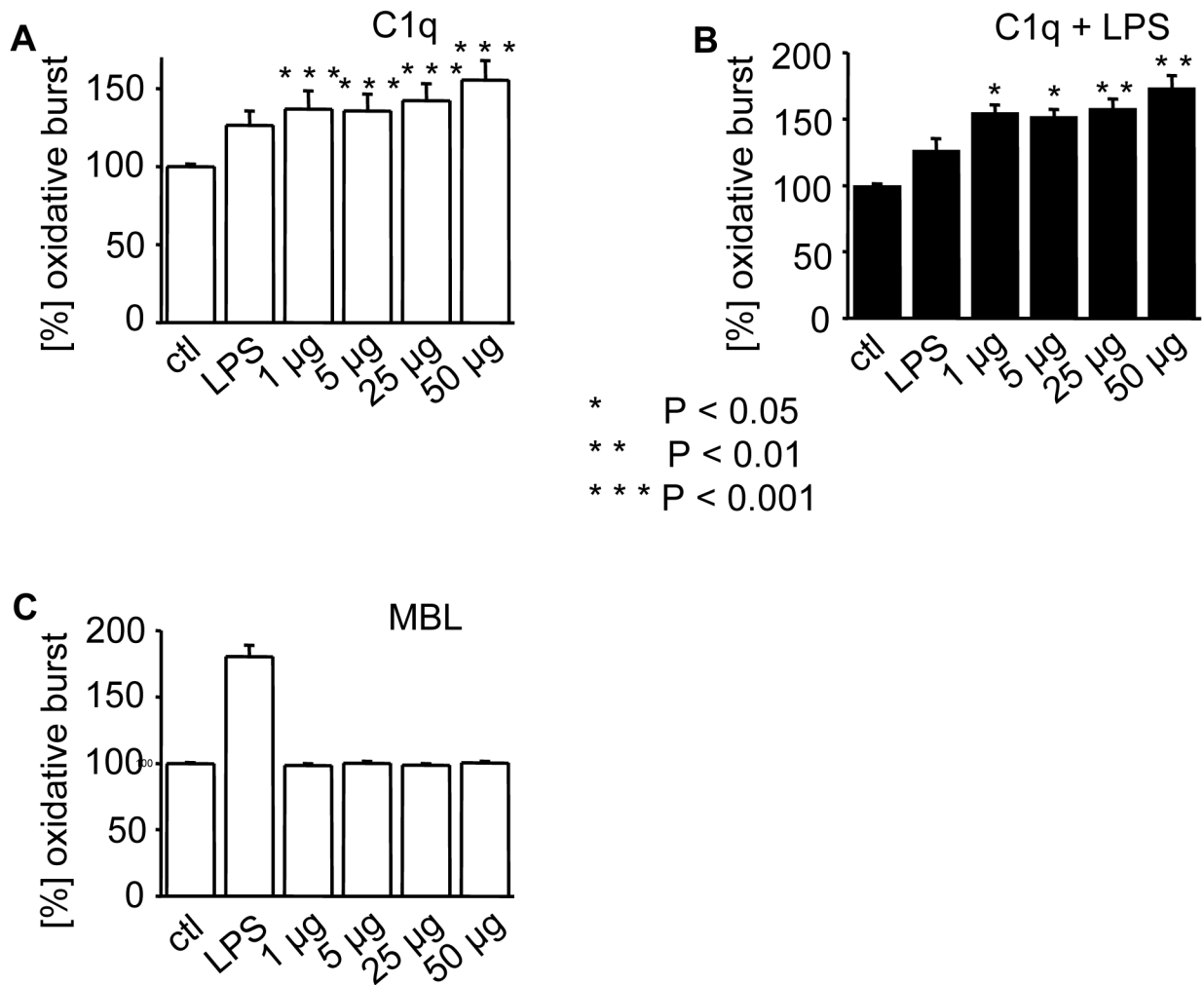


Figure 4.

C1q induces oxidative burst in microglial cells, but MBL is ineffective.

Microglia cells were stimulated with C1q (A, B, 1 to 50 μg/ml) and MBL (C, 1 to 50 μg/ml) alone and C1q in combination with LPS (100 ng/ml) over 24 h. The oxidative burst reaction was measured by colourimetric reaction of dihydrofluorescein. Data are mean ± SEM summarized from three independent experiments.

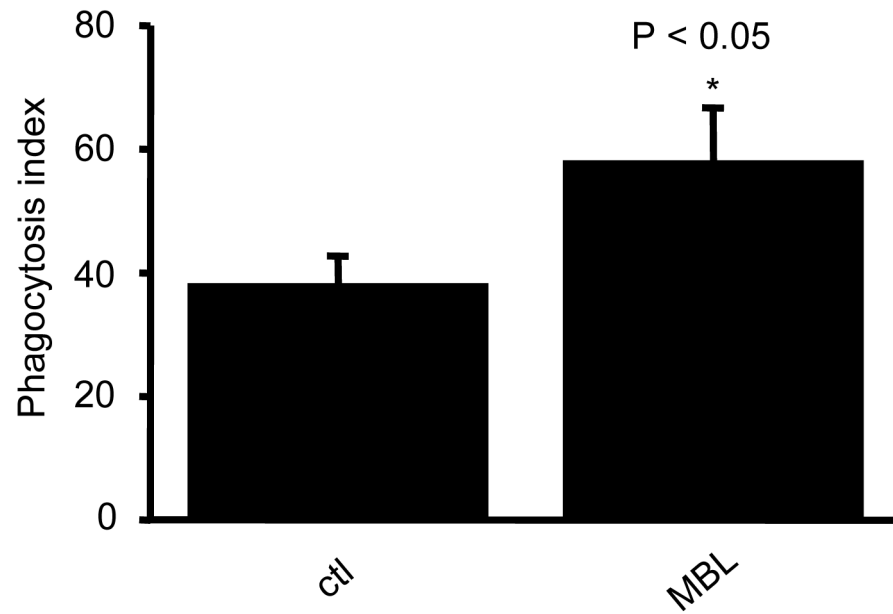


Figure 5.
MBL induces microglial phagocytosis.
Microglial cells were stimulated with MBL (50 μ g/ml) or DMEM/10 % FCS as negative control for 30 min at 37°C in presence of obsonized phagocytose microparticles (10^8 particel/ml). Phagocytotic index was calculated based on phagocytosing activity.

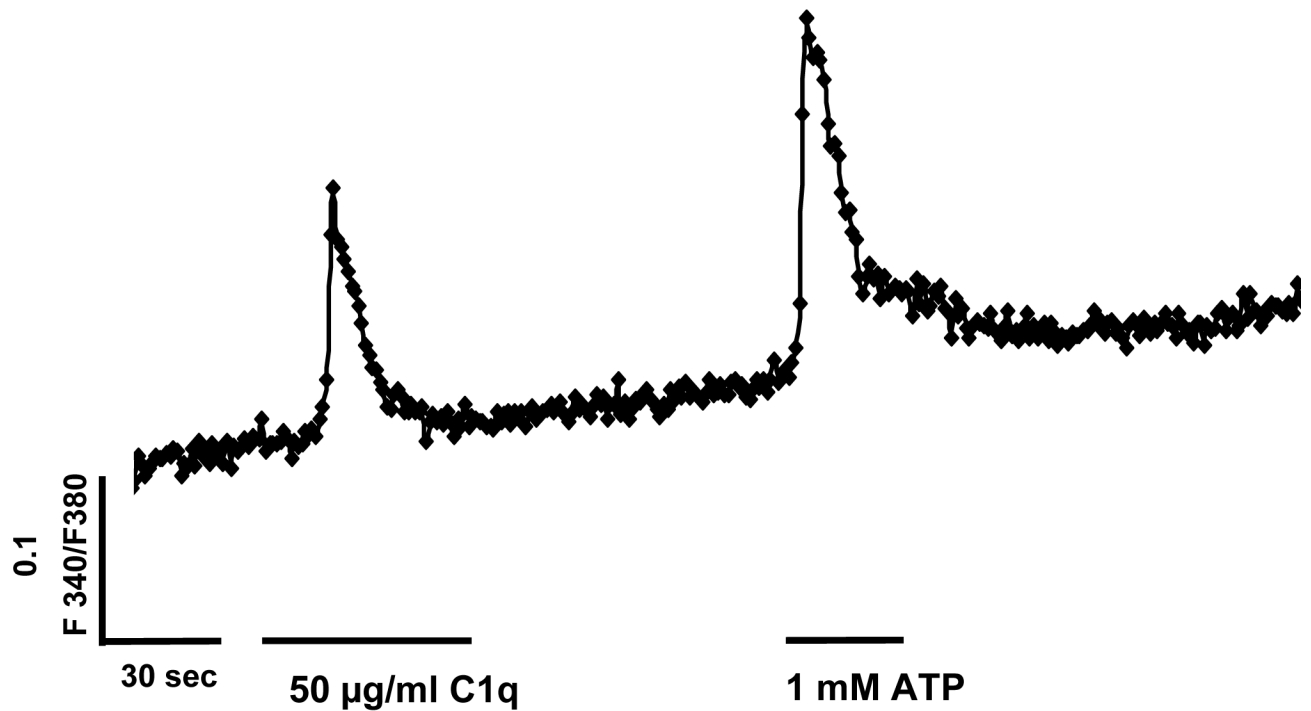


Figure 6.

C1q induces intracellular Ca^{2+} changes in microglial cells.

Cultured rat microglia were loaded with Fura-2 AM and were stimulated with rat C1q (50 µg/ml) for 30 sec by using a local application pipette. 2 min after C1q stimulation, 1 mM ATP was applied for 30 sec to test cell viability. The graph shows a characteristic microglial Ca^{2+} response from (n=179). The ratiometric images (340/380 nm) were taken every second during drug exposure.